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Salivary antigens of the cat flea, Ctenocephalides felis-felis.
Lee SE, Jackson LA, Opdebeeck JP.

2) Presence of calreticulin in vector fleas (Siphonaptera)
Jaworski, D. C.; Higgins, J. A.; Radulovic, S.;
Journal of Medical Entomology, (1996) Vol. 33, No. 3, pp. 482-489.

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Salivary antigens of the cat flea, *Ctenocephalides felis felis*

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SUMMARY

The cat flea, Ctenocephalides felis felis, *is the major cause of flea bite hypersensitivity (FBH) in dogs and cats, yet little progress has been reported on identifying the antigens responsible. We obtained flea salivary antigens by washing secretions from containers probed by the mouthparts of fleas, and by extracting whole flea salivary glands. Mice were exposed to feeding fleas to generate antibodies to salivary antigens injected* in vivo. *The sera were tested for antibodies against the salivary antigens described and against a whole flea extract; in indirect ELISA, antibodies to salivary secretions were detected in 60% of the sera from mice exposed to feeding fleas. These sera identified four protein bands at apparent MW 56, 54, 42 and 40 K which corresponded to prominent protein bands in whole salivary gland extracts identified by protein staining after SDS-PAGE. Fixed sections of whole fleas exposed to the antisera showed that only structures within the salivary glands were identified. The salivary secretions and gland extracts are now being used to study immune responses of dogs suffering from FBH.*

Keywords *cat flea,* Ctenocephalides felis felis, *saliva, flea bite hypersensitivity, flea allergy dermatitis, intradermal skin testing, FBH, FAD, IDT*

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INTRODUCTION

The cat flea, *Ctenocephalides felis felis*, is acknowledged worldwide as the major cause of flea bite hypersensitivity (FBH) in dogs and cats (Dryden & Rust 1994). In spite of this, little progress has been reported since the early 1960s on the identification of the flea antigens responsible for inducing the hypersensitive response. Benjamini *et al.* (1963) observed that unfed fleas probed and released oral secretions onto a variety of surfaces. Their experiments with the collected oral secretions suggested that the substances responsible for the bite reaction in the guinea pig host were haptenic in nature. It was subsequently postulated that haptens from flea saliva associate with collagen in the host skin to attain full allergenicity (Michaeli *et al.* 1966). The early studies on the allergenicity of flea oral secretions (Benjamini *et al.* 1960, 1963, Young *et al.* 1963 and Michaeli *et al.* 1966) preceded establishment of discontinuous electrophoresis and immunoblotting techniques; hence, individual allergens were not identified and conclusions about the allergenicity of different fractions were based on skin tests in sensitized hosts. In later studies of FBH complex whole flea extracts were examined by electrophoresis and immunoblotting techniques (Halliwell, Preston & Nesbitt 1987, Greene *et al.* 1993, Stolper & Opdebeeck 1994, McKeon & Opdebeeck 1994), but whole flea extracts contain many proteins that may be irrelevant to the hypersensitive response to flea bites. Extracts of salivary antigens are likely to be a better source of antigen for identifying particular allergens.

We generated antibodies to flea saliva in mice by exposing them to feeding fleas, and tested the reactivity of the mice sera with flea saliva collected *in vitro*, with extracts of whole salivary glands and with a soluble whole flea extract (FS) (Stolper & Opdebeeck 1994), using ELISA and immunoblots. We also tested the specificity of the sera for antigens in the salivary gland using immunohistochemistry. The work described was preliminary to developing assays to detect flea allergens, using sera from dogs with and without FBH.

MATERIALS AND METHODS

Mice

Twenty-nine adult female Quackenbuch mice (eight weeks

old) were used in this study; 20 as test animals and nine as controls. The mice were obtained from the specific pathogen free unit of the Central Animal Breeding House, The University of Queensland, and were free of mites.

Fleas

Cat fleas (*Ctenocephalides felis felis*) were raised and harvested using the system described previously (Lee, Johnstone & Opdebeeck 1995). Fleas used in all experiments were ≤3 days post emergence.

Exposure of mice to feeding fleas

All mice were anaesthetized using an appropriate dosage of xylazine (Parnell Laboratories (Aust) Pty Ltd) and ketamine (Ketapex, Apex Laboratories Pty Ltd) injected intraperitoneally (Green 1981) and the abdomens were closely shaved. About 150 unfed fleas were placed in a tube (13 × 100 mm) and sealed in with fine gauze. Test mice were placed dorsally and the tube containing fleas was clamped in position over the shaved region, with the gauze tightly apposed to the skin, for 20 min. Mice were exposed to fleas daily for four consecutive days each week for seven consecutive weeks; in total the mice were exposed to approximately 1400 flea feeding hours (28 days × 0·3 h × 150 fleas). Mice were then anaesthetized, bled by cardiac puncture and killed. Serum was collected and stored at −20°C. Control mice were treated as above except they were not exposed to fleas.

Antigens

Four flea extracts were prepared

A. Salivary Secretions (SAL)

Unfed adult fleas were placed in a wide mouth container and cooled at −20°C for 15 min to temporarily immobilize them. 10–20 chilled fleas were transferred into each well of a microtitre plate (96 well, Disposable Products) and were sealed in place in the wells by inverting an identical microtitre plate over the original and affixing with a rubber band. The plates were incubated at 37°C for six h with inversion every 15 min to allow the fleas to probe the surface of both plates. Flea faecal antigens were considered to be negligible based on a report that no case of defaecation was seen in several thousand unfed fleas observed for up to 6 h (Benjamini *et al.* 1963). Fleas were removed after 6 h and the plates stored at −70°C.

B. Lyophilized salivary secretions (SAL-LYO)

Approximately 200 unfed fleas were incubated in a 50 mL polypropylene tube for eight h at 25°C. The fleas were removed and the surface was washed with 0·17 M ammonium carbonate. Washes from several tubes were pooled, the protein concentration of the wash was determined (Bio-Rad Protein Assay kit) and the solution was lyophilized. Before use, the powder was reconstituted to 1 mg/mL with buffer (0·15 M PBS, pH 7·2, containing 1 mM EDTA).

C. Salivary gland extracts (SG)

Salivary glands, including ducts, were dissected from adult fleas submerged in a drop of distilled water. The glands were transferred into SDS-reducing buffer (Laemmli 1970) and vortexed, then stored at −20°C. The protein content of salivary gland extract was determined as ~0·2 μg per gland by use of the Bio-Rad *DC* Protein Assay kit adapted to a microtitre plate format.

D. Soluble whole flea extract (FS)

Soluble antigens were extracted from whole fleas using the method of Stolper and Opdebeeck (1994). Briefly, about 4000 fleas were homogenized in 0·15 M PBS pH 7·2, sonicated and centrifuged at 15 000 *g* for 20 min and the resultant supernatant centrifuged at 100 000 *g* for one h to yield a supernatant designated FS. The supernatant was stored at −70°C. The protein concentration of FS was determined using a modified Lowry method (Markwell *et al.* 1978).

Indirect ELISA to measure antibodies to flea antigens

100 μL 0·06 M carbonate buffer pH 9·6 was added to each well of the SAL plates prepared as described above, and the plates were incubated for 16 h at 4°C. For ELISA using FS, wells of microtitre plates were each coated with 100 μL of FS (5 μg/mL in carbonate buffer) and incubated for 16 h at 4°C. Plates were washed and blocked with 5% low fat milk powder in PBS (5% LFM-PBS) then mice sera diluted 1:30 were added. Goat anti-mouse IgG (whole) conjugated to horse-radish peroxidase (Cappel) was used as the marker antibody. The substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) was used to develop the assay which was read at 405 nm. Mice sera were tested in either five replicates against SAL or three replicates against FS. To determine whether a mouse serum had antibodies to the flea antigens, a positive threshold value was calculated from the mean absorbance readings at 405 nm for the naive mice, plus two standard deviations. The correlation between antibody levels against SAL and FS was calculated using a Microsoft Excel statistical analysis programme.

Characterization of antigens using SDS-PAGE

SAL-LYO was reconstituted with 0·15 M PBS containing

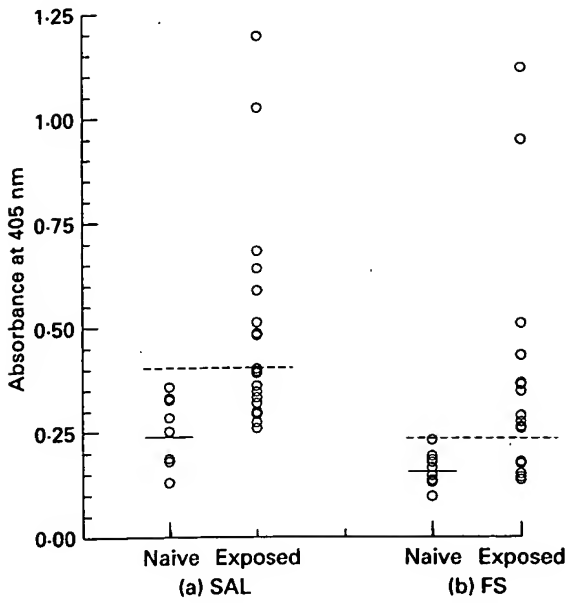

Figure 1 Antibodies against: (a) salivary antigens (SAL) and (b) soluble whole flea extract (FS) in sera of flea-naive mice and mice exposed to feeding fleas. Antibodies were measured by indirect ELISA and are shown as absorbance units at 405 nm. ○: values of individual mice sera, —— mean value for flea-naive mice sera; - - - - plus 2 SDs for mean value for flea-naive mice sera.

1 mM Na_2EDTA pH 7·2 to a protein concentration of ~1 mg/mL. Proteins from FS, SAL-LYO and SG were separated on denaturing, reducing polyacrylamide gels (10% or 12% separating gel and 4% stacking gel) (Laemmli 1970) prepared in a Bio-Rad Mini-PROTEAN II gel system. Separated proteins were stained with silver (Bio-Rad Silver Stain Plus kit).

Western blots

FS, SAL-LYO and SG proteins were separated by SDS-PAGE as above and transferred electrophoretically onto nitrocellulose paper (NCP) (Amersham Hybond-C extra) (Towbin *et al.* 1979). After transfer, the NCP was placed in PBS for 16 h to renature proteins (Birk and Koepsell 1987), the membrane was blocked with 5% LFM-PBS and then diluted mice sera (1:200) were applied to the NCP. After incubation and washing, goat anti-mouse IgG (whole) serum conjugated to horse-radish peroxidase (Cappel) was added. The blot was developed using a Pierce ImmunoPure DAB-Metal enhanced substrate kit.

Immunolabelling of flea sections

Adult fleas were cooled in a freezer to immobilize them. Their legs were severed to promote fixative penetration, and

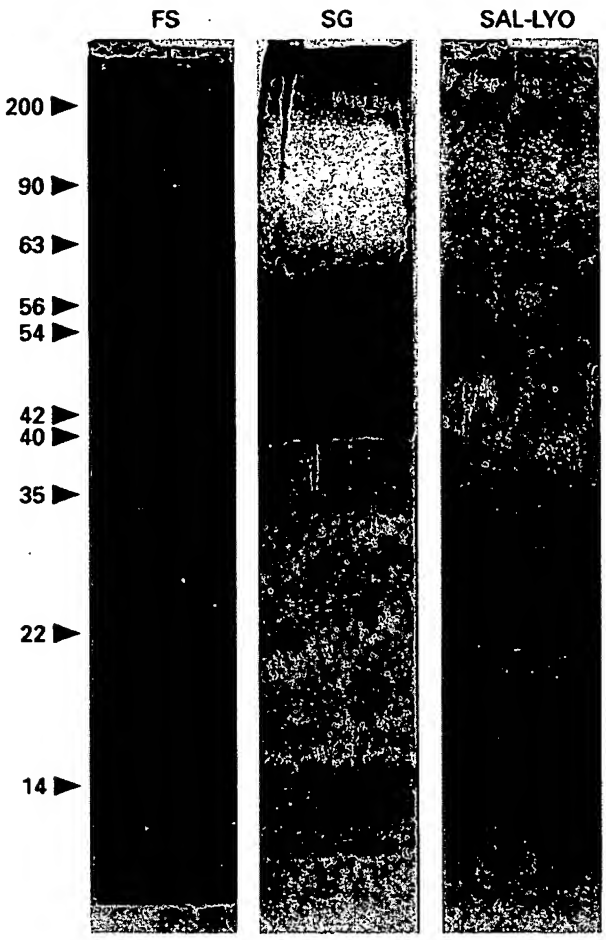

Figure 2 Proteins identified in soluble extract of whole fleas (FS), flea salivary gland extracts (SG) and flea salivary secretions (SAL-LYO) using SDS-PAGE (10% gel; silver stain).

the fleas were then placed in fixative (2·5% glutaraldehyde in 0·1 M cacodylate buffer pH 7·4) for at least three h at 4°C. The fixed fleas were processed and embedded in LR White resin (Newman & Hobot 1987). Semi-thin parasagittal sections (1 μm) were cut using a glass knife and an Ultramicrotome (Huxley) and were transferred onto silanized slides (Dako). Every tenth section was stained with toluidine blue (0·1% in 0·1% sodium tetraborate) and examined under a compound microscope until a section containing salivary glands was located. This stained section was used to identify structures and the serial sections subsequent to this were used for immunolabelling. Protein A-colloidal gold (PA-Au) with 9 nm particle size for use in immunogold silver staining (IGSS) was prepared according to the method of Slot & Geuze (1985). The serial sections were etched (Stenzel 1987), blocked and incubated with mouse serum diluted 1:50. After washing, the sections were incubated with PA-Au. The gold staining was then silver enhanced as

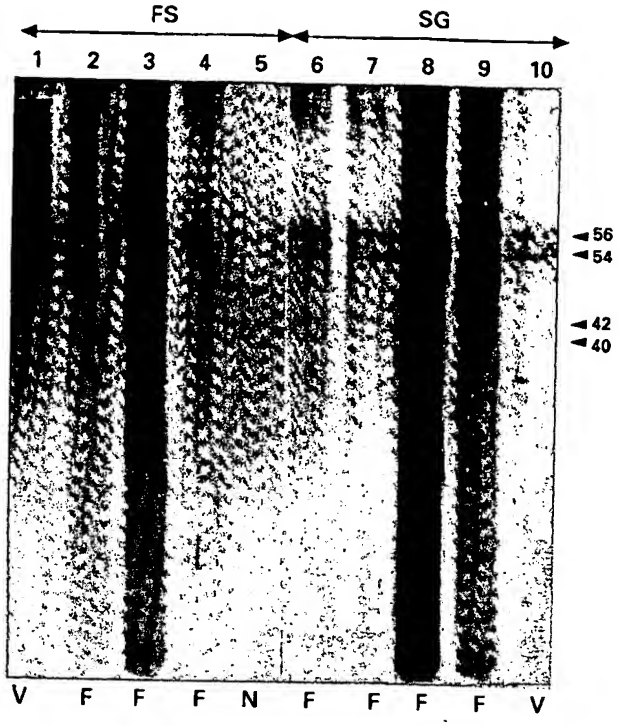

Figure 3 Western blot showing those antigens in soluble whole flea extract (FS, lanes 1–5) and in flea salivary gland extracts (SG, lanes 6–10) that are recognized by antibodies in mice sera. Lanes 1 and 10 were incubated with sera from mice vaccinated with FS; lanes 2–4 and 6–9 with sera from mice exposed to feeding fleas; and lane 5 with serum from a flea-naive mouse. The results shown here and in Figure 4 are from a single gel and blot in each case. Two lanes that displayed data irrelevant to this paper were cut from the original photograph which was reassembled and rephotographed.

described in the literature provided with the Sigma Silver Enhancer Kit for use in IGSS.

RESULTS

Detection of antibodies in mice to SAL, in ELISA

Eight of the 20 sera collected from mice exposed to 1400 flea feeding hours had positive but variable levels of antibodies against SAL using a positive threshold of $A_{405nm} > 0{\cdot}410$ (Figure 1a). Sera from 12 of the mice exposed to fleas had antibodies against FS using a positive threshold of $A_{405nm} > 0{\cdot}237$ (Figure 1b). The levels of antibody against SAL and FS were significantly positively correlated ($r = 0{\cdot}8$; $P < 0{\cdot}001$).

Proteins identified in FS, SAL-LYO and SG using SDS-PAGE (Figure 2)

FS displayed numerous bands of protein ranging from

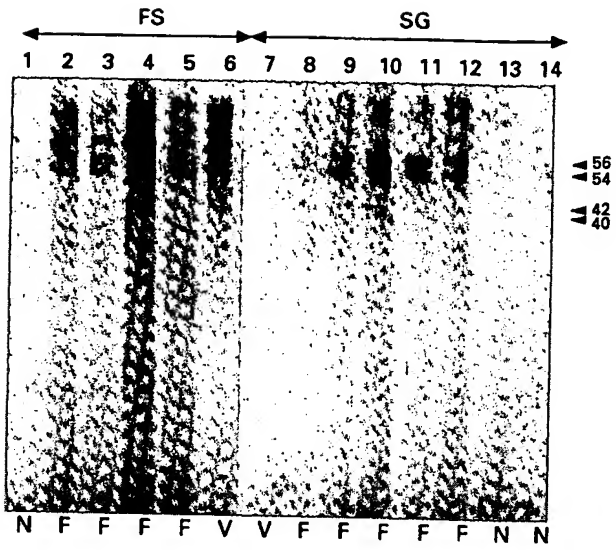

Figure 4 Western blot showing those antigens in soluble whole flea extract (FS, lanes 1–6) and in flea salivary gland extracts (GS, lanes 7–10) that are recognized by antibodies in mice sera. Lanes 6 and 7 were incubated with sera from mice vaccinated with FS; lanes 2–5 and 8–12 with sera from mice exposed to feeding fleas; and lanes 1, 13 and 14 with serum from a flea-naive mice. The results shown are from a single gel and blot.

MW<14 to >200 K following SDS-PAGE. The majority of prominent bands in SG were at MW 40–56 K and some less prominent bands existed at MW<18 K; other faint bands were also present. SAL-LYO had a prominent band at MW 14 K, a series of bands from MW 22–35 K, and a distinct band at MW 54 K.

Antigens identified in FS, SAL-LYO and SG by sera from mice exposed to feeding fleas

Multiple bands with MW>40 K, including a prominent band at MW 54 K, were recognized in FS by antisera from the mice which reacted to feeding fleas (Figure 3, lanes 2–4; Figure 4, lanes 2–5). In SAL-LYO, 4/8 of these positive mice recognised bands at MW 54 and 56 K and these two bands were consistently recognised in SG also (Figure 3, lanes 6–9; Figure 4, lanes 8–12). The band at MW 56 K was barely detected in FS. Other bands at MW 40, 42 K (Figure 3, lanes 8 and 9; Figure 4, lanes 9 and 10) and MW<14 K and four bands of MW 40–56 K were also variably recognised in SG by individual mice exposed to feeding fleas. Sera from naive mice showed low levels of recognition of high molecular weight bands in FS (Figure 3, lane 5; Figure 4, lane 1) and barely detectable recognition of bands in SG (Figure 4, lanes 13 and 14).

Immunolabelling

Dissected flea salivary glands (4 per flea) are shown in

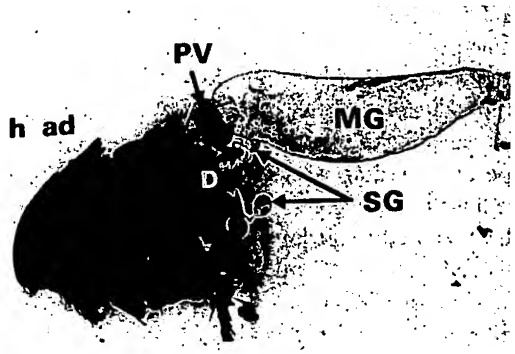

Figure 5 Salivary glands (SG) in a dissected adult female flea. The glands are connected with the mouthparts by cuticle-lined salivary ducts (D). PV, proventriculus; MG, midgut. Magnification ×70.

Figure 5. These are located in the abdomen of the flea and are connected to the mouth parts by cuticle-lined salivary ducts (Rothschild, Ito & Schlein 1986). A section of salivary gland of an adult female flea stained with toluidine blue is demonstrated in Figures 6a and b. The gland consists of a single layer of epithelial cells surrounding a central lumen. Antibodies from a mouse exposed to feeding fleas selectively immunolabelled the epithelial cells of the salivary gland (Figures 6c and d). No other organs in the whole feal section reacted with the antibodies.

DISCUSSION

Mice exposed to feeding fleas developed antibodies that recognised components of both a whole flea extract (FS) and secretions (SAL) deposited by unfed fleas on a plastic surface. The responses to both antigens, measured by ELISA, were significantly correlated. This implies that the same antigens were being recognized in both SAL and FS. However FS detected a greater number of positive responses in the group of mice exposed to feeding fleas, suggesting that the materials secreted during probing on an inert surface may be present at a level which is below the optimum for an ELISA, thus affecting the sensitivity of the assay. The amount of protein antigen secreted by ~15 fleas in a microtitre well is low; the total quantity was found to be

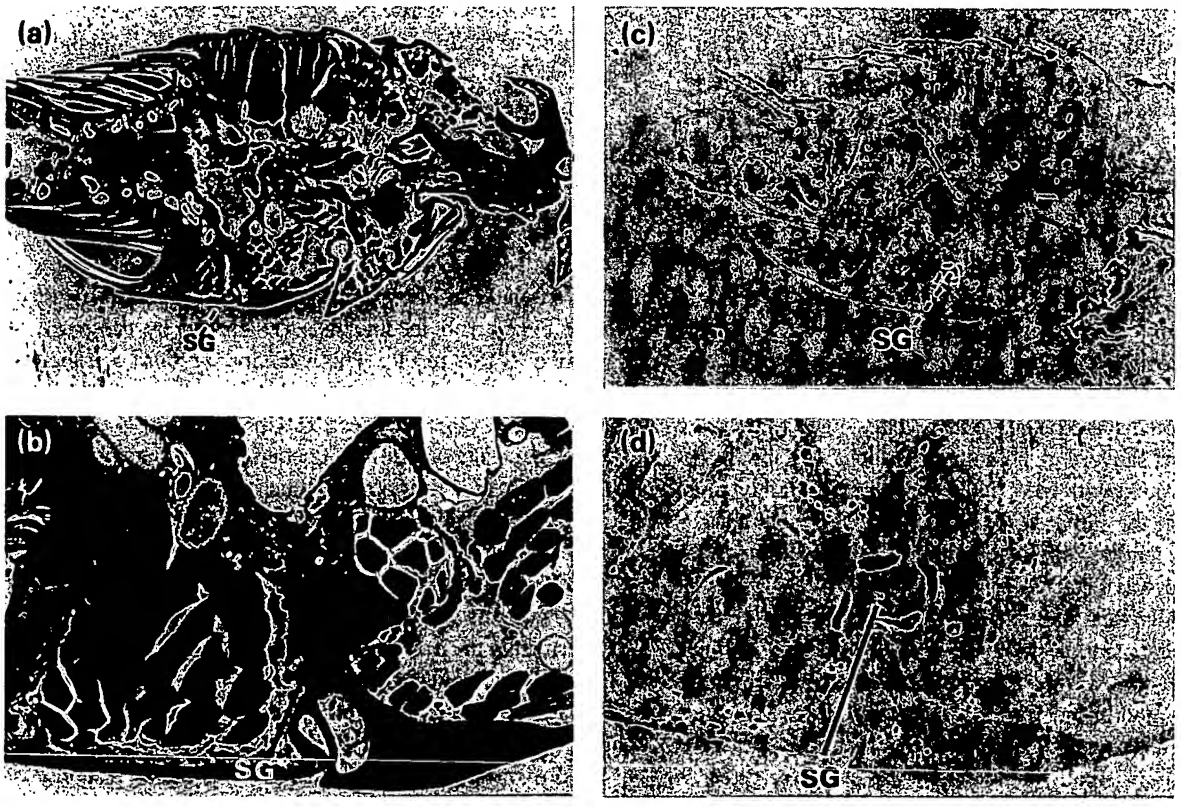

Figure 6 (a) and (b) Parasagittal section of an adult female flea, showing a cross section of a salivary gland in the lower abdomen. The single layer of epithelial cells of the salivary gland surround the central lumen. Toluidine blue stain. SG, salivary gland. (a) Magnification ×70; (b) Magnification ×280. (c) and (d). Immunolabelling of a serial section subsequent to the section in (a). The unstained section was exposed to serum from a mouse exposed to feeding fleas and antibodies attaching to the section were detected using protein A-colloidal gold with silver enhancement. The epithelial cells of the salivary gland are clearly positive. (c) Magnification ×70; (d) Magnification ×280.

<1 μg per well using the Bio-Rad protein assay, and most published ELISA use ≥1 μg protein per well. Some enzymatic degradation of SAL also may have occurred between deposition and freezing of plates preparatory to ELISA. Alternatively, salivary secretions collected by allowing fleas to probe plastic may lack antigens injected by fleas feeding *in vivo*.

The protein band patterns of SAL-LYO and SG after electrophoresis differed and few of the prominent bands coincided. Secreted salivary antigens represent only a portion of a whole salivary gland extract. To obtain sufficient secreted salivary antigen for use in gels and immunoblots many hundreds of fleas were allowed to probe polypropylene over eight h, and the saliva components washed from the plastic were lyophilized. Salivary components with particular affinity for plastic may not therefore be represented in SAL-LYO and some enzymatic degradation of antigens may have occurred in the period between antigen deposition and washing. An antigen at MW 56 K, for example, was present at low concentration in SAL-LYO; it was difficult to identify this protein in stained SDS-PAGE but evidence for its presence was obtained subsequently in immunoblots. This molecule may be highly immunogenic when deposited in the host, or may be removed or damaged in the *in vitro* product. The ducts of salivary glands are lined with cuticle which contains proteins that can be extracted with detergents (Anderson 1979, Phillip & Rumjaneck 1984) and salivary gland extracts would contain some cuticular components in addition to somatic components of the epithelial cells. After dissection from a live flea, the salivary glands were immediately extracted in sample buffer which contains denaturing and reducing agents that are likely to inhibit enzymatic activity, and the extracts were stored frozen at −20° C. Components in SG extracts contrasted with those in SAL-LYO in having large concentrations of proteins at MW 40–56 K. This suggests that these are abundant salivary gland antigens which, with the exception of a protein with MW 54 K, are secreted in only small quantities in the saliva secreted *in vitro*. A protein at MW 54 K was identified in FS by protein staining after SDS-PAGE, and was detected in that extract by sera from the mice on which fleas had fed. There was little evidence for a protein component at MW 56 K in FS. These findings suggest that the MW 54 K protein is abundant in extracts of salivary glands and whole fleas, whereas the MW 56 K protein is an abundant salivary gland antigen which is present also in small quantities in the saliva secreted *in vitro* but represents only a small portion of a whole flea extract. Barely detectable recognition of some bands in FS and SG by sera from naive mice may be non-specific binding, or FS in particular may contain some cross reactive antigens which are not unique to the flea.

In general we considered the salivary gland extract to be a more comprehensive antigen preparation which better reflected the antigens injected into the host by a feeding flea than did salivary secretions washed off a solid phase. The specific labelling of salivary glands by the serum of a mouse exposed to feeding fleas shows that reactivity of the host to feeding fleas is against antigens from the salivary glands. Mice exposed to feeding fleas recognized bands in SG at MW 54 and 56 K and, in addition, some mice recognized bands at MW 40 and 42 K. These four antigens may prove to play a causal role in FBH in dogs although we saw no evidence of hypersensitivity in mice exposed to biting fleas over seven weeks. The flea antigens described are either the same or similar in MW to antigens as MW 42, 45 and 55 K described by Halliwell *et al.* (1987) as being recognized by serum from a flea allergic dog but not by serum from a flea-naive dog, and a component of MW 40 K present in whole flea extracts than Greene *et al.* (1993) suggested is worthy of major allergen status.

Components with a MW <40 K are present in both SG and SAL-LYO, but only one of the mice exposed to biting fleas developed antibodies to these. The low MW components may be haptens, or are not significantly recognized by mice exposed to feeding fleas. Halliwell *et al.* (1987) found that the low MW components of a fractionated commercial flea extract were irrelevant to the IgE immune response in a small group of dogs with FBH. They tested the allergenicity of different MW fractions by performing intradermal tests (IDT) on dogs and found no reaction in 6 dogs with FBH to a dialysate with MW <10 K, but identified a major allergenic fraction with MW >30 K.

We found considerable variation in the immune response of mice exposed to feeding fleas, evidenced by the range of positive ELISA results. This variation was also a feature of previous work where antibody levels to flea antigens were measured in dogs (McKeon & Opdebeeck 1994), although longer exposure or a different pattern of exposure to the biting fleas might give rise to more consistent levels of antibodies.

The recognition of bands at MW 56, 54, 42 and 40 K by antibodies in the sera of mice exposed to fleas implies that these bands may be important antigens secreted by biting fleas and this hypothesis is supported by previous work described in the literature (Halliwell *et al.* 1987, Green *et al.* 1993). Salivary gland extracts proved to be a better source of these antigens than the oral secretions collected by fleas probing plastic surfaces. In addition, response to salivary gland extracts compared to the more complex response of FS suggests that the salivary gland extract is better able to identify relevant and specific antibody responses. We are currently sequencing the four salivary molecules identified and assessing their role in FBH using IDT in allergic and non-allergic dogs.

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